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Overlapping genes produce proteins with unusual sequence properties and offer insight into *de novo* protein creation

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Abbreviations

Only abbreviations used in the main text are listed here; others are given in the figure captions.

30K, conserved domain of the 30K family of movement proteins; aa, amino acid; dsRNA, double-stranded RNA; GT, guanylyltransferase; MP, movement protein; MT, methyltransferase; N, nucleoprotein; NSs, non structural protein of *orthobunyaviruses*; ORF, open reading frame; PDB, protein databank (database of protein structures); PFAM, Protein families (database of families of protein sequences); ssRNA, single-stranded RNA; TGB, triple gene block; RE, relative (compositional) entropy; tm, transmembrane segment.

Abstract

It is widely assumed that new proteins are created by duplication, fusion, or fission of existing coding sequences. Another mechanism of protein birth is provided by overlapping genes. They are created *de novo* by mutations within a coding sequence that lead to the expression of a novel protein in another reading frame, a process called "overprinting". To investigate this mechanism, we have analyzed the sequences of the protein products of manually curated overlapping genes from 43 genera of unspliced RNA viruses infecting eukaryotes. Overlapping proteins have a sequence composition globally biased towards disorder-promoting amino acids and are predicted to contain significantly more structural disorder than non-overlapping proteins. By analysing the phylogenetic distribution of overlapping proteins, we were able to confirm that 17 of these had been created *de novo* and to study them individually. Most proteins created *de novo* are orphans (*i.e.* restricted to one species or genus). Almost all are accessory proteins that play a role in viral pathogenicity or spread, rather than proteins central to viral replication or structure. Most *de novo* proteins are predicted to be fully disordered and have a highly unusual sequence composition. This suggests that some viral overlapping reading frames encoding hypothetical proteins with highly biased composition, often discarded as non-coding, might in fact encode proteins. Some *de novo* proteins are predicted to be ordered, however, and whenever their 3D structure has been solved, it corresponds to a fold previously unobserved, suggesting that their study could enhance our knowledge of protein space.

Introduction

Since their discovery (76), overlapping genes, i.e. DNA sequences simultaneously encoding two or more proteins in different reading frames, have exerted a fascination on evolutionary biologists. Among several mechanisms, they can be created by a process called “overprinting” (43), in which a DNA sequence originally encoding only one protein undergoes a genetic modification leading to the expression of a second reading frame in addition to the first one (Fig. 1). The resulting overlap encodes an ancestral, "overprinted" protein region, and a protein region created *de novo* (i.e. not by duplication), called an "overprinting" or "novel" region (Fig. 1). At present, it is widely thought that the creation of proteins *de novo* is very rare, contrary to their emergence by gene duplication, which is thought to be the major factor (for reviews, (55, 94)). However, this belief might actually reflect the fact that proteins created *de novo* are in general very difficult to identify (55). Indeed, a long-standing question is whether a protein that has no detectable homolog in other organisms (called an "orphan" protein or “ORFan” (27) or “taxonomically restricted” (110)) represents a protein created *de novo* in a particular organism, or merely a protein that is a member of a larger family whose other members have diverged beyond recognition or become extinct (115). Proteins created *de novo* by overprinting provide a valuable opportunity to address these questions, and this constitutes one of the two strands of our study.

Practically all studies on overlapping genes have been focused on evolutionary constraints and informational characteristics at the DNA level (e.g. (46, 71, 75, 84, 85, 114)). However, very little has been done to assess potential effects of the overlap on the corresponding protein products. Two studies reported that overlapping proteins are enriched in amino acids (aa) with a high codon degeneracy (arginine, leucine and serine) (68) and that they often simultaneously encode a cluster of basic aa in one frame and a stretch of acidic aa in the other frame (66).

The other strand of the present study is based on earlier observations of the overlapping gene set of measles virus (41), which suggested that protein regions encoded by overlapping genes might have a propensity towards structural disorder.

Structural disorder is an essential state of numerous proteins, in which it is associated mostly with signalling and regulation roles (21, 96, 111). The key feature of intrinsically disordered proteins (also called “unstructured” or “natively unfolded”) is that under physiological conditions, instead of a particular 3D structure, they adopt ensembles of rapidly inter-converting structural forms. Different degrees of disorder exist, from random coils to molten globules (100), and some disordered regions can become ordered under certain conditions (21, 96, 117). A variety of computer programs have been developed to predict these regions (19, 23, 101)). Each predictor typically differs on what kind of “disorder” it identifies (23, 78), matching only some of the types of disorder mentioned above. Therefore, in order to choose a proper

predictor, it was necessary to define precisely what kind of structural disorder we expected to find in proteins encoded by overlapping genes.

At least 2 non-exclusive hypotheses can explain why overlapping genes might encode disordered proteins: 1) the newly created (overprinting) protein of each overlap might tend to be disordered; 2) structural disorder in proteins encoded by overlapping genes might alleviate evolutionary constraints imposed on their sequence by the overlap. These hypotheses are clarified below.

Intuitively, the conditions required for a protein to fold into a stable 3D configuration, including sequence composition, periodicity and complexity, are such that structurally ordered proteins represent a vanishingly small fraction of all possible aa sequences. Indeed, proteins artificially created from random nucleotide sequences generally have a low secondary structure content (107, 112). Hence our hypothesis 1): novel, overprinting proteins are not expected to have a fixed 3D structure at birth, given the low probability of generating structure from a completely new sequence.

Disordered proteins are generally subject to less structural constraint than ordered ones (13). Hence hypothesis 2): the presence of disorder in one or both products of an overlapping gene pair could greatly alleviate evolutionary constraints imposed by the overlap, allowing both protein products to scan a wider sequence space without losing their function.

Both hypotheses only suppose the lack of a rigid structure, as opposed to a total lack of structure (e.g. some proteins created *de novo* from a random nucleotide sequence, though lacking secondary structure, have a certain degree of order (112)). For that reason, in this work, we use the widest possible definition of disorder, i.e. lack of a rigid 3D structure, and we use a program whose predictions of disorder correspond to this definition, PONDR VSL2 (69) (see Results section).

In this work, we collected a large number of experimentally proven cases of proteins encoded by overlapping genes in unspliced eukaryotic RNA viruses and analyzed their sequence properties.

Material and Methods

Selection and curation of the dataset of viral overlapping gene products

We set out to find virus genomes containing overlapping genes whose existence was supported by experimental evidence. We first downloaded the file "Virus.ids", release July 2nd 2004 (ftp://ftp.ncbi.nih.gov/genomes/IDS/Viruses.ids) containing accession numbers for all complete viral genomes (except those of bacteriophages) from the NCBI viral database (6). We then downloaded the 1562 corresponding genomes or genome segments, corresponding to 1098 viruses (some viruses have a segemented genome), and parsed all relevant information for each genome. Since the NCBI viral genome database (6) is not completely reliably annotated (62), we had to carefully select *bona fide* overlapping genes. We excluded from the analysis all files containing a "join" instruction (regardless whether it reflected a splicing event, a frameshift, or a circular genome with genes crossing the genome map borders) because their manual curation would have been too time-consuming. We excluded from the analysis all DNA viruses, all viral genera in which at least one virus is known to make use of splicing and selected only overlaps longer than 90nt, corresponding to 30aa (see Results). We considered only one prototype virus per genus. We kept overlaps only if there was biochemical evidence that both proteins they encoded existed (*i.e.* detection in infected cells, or in *in vitro* translation experiments), or if such evidence was available for the protein products of a homologous gene overlap in a related virus.

Overlaps found only in one virus species might stem from a sequencing error resulting in an artefactual N-term or C-term extension. Therefore, we checked in the literature that the proteins expressed had the actual, predicted size, or that several viral strains from that species also had a similar overlap. If we could not exclude a sequencing artefact, we discarded the overlap.

If the theoretical start or stop codon of an overlapping ORF as described in the NCBI file was incorrect, it was manually corrected (for instance VP5 of infectious pancreatic necrosis aquabirnavirus starts at nucleotide 113 and not 68 (108)). A few unspliced RNA viruses contain *bona fide* overlapping genes that are not described in the corresponding NCBI genome file. They were included in the analysis and the missing protein they encode was manually added: rice dwarf phytoreovirus OP-ORF (89), Theiler's cardiovirus protein L* (104), and vesicular stomatitis Indiana vesiculovirus protein C' (47). We provide their sequence in Supplementary File S1.

A few viruses make use of frameshifting to generate overlapping reading frames but (presumably by mistake) their genome file does not contain a "join" instruction (for instance the mumps rubulavirus P/V overlap) and therefore were included in the analysis. Among those, some frameshifts or editing events result in genes that are partially colinear (upstream of the frameshift) and that thus truly overlap only downstream of the frameshift. In these cases, we excluded the colinear part. For instance, in the case of the mumps rubulavirus P/V gene system we excluded the N-terminal part common to both P and V (41). Finally, in

some cases an ORF (called "1") overlaps several ORFs (called 2, 2', 2'', 2''', etc.) that are colinear which each other, because of alternative translation initiation sites, for instance proteins C, C', Y1 and Y2 in Sendai respirovirus (16)). In that case we kept only the ORF 2 for which the overlap with ORF 1 is the longest (in that case the ORF C).

Viral taxonomy

Viral taxonomy changes fast and some names of viral taxons that are widely used by virologists are not officially recognized. Several of these taxons proved crucial to interpret our results in an evolutionary light (*e.g.* the proposed family *Tubiviridae* (97)). Therefore, in addition to the official taxonomy (58), we have also indicated proposed taxa, indicating the corresponding references. The reader interested can consult the web site where proposals are made to the International Committee for the Taxonomy of Viruses: http://talk.ictvonline.org.

PONDR analysis of viral genes

The sequences of overlapping genes and their protein products were stored in a MySQL database for analysis. Protein intrinsic disorder was predicted using PONDR® VSL2 (69), a neural network trained on a set of ordered and disordered sequences, which relies on attributes such as composition of particular aa or hydropathy to predict disorder propensity along a protein sequence. PONDR predictions were also stored in the database.

Bootstrapping was used on the results to generate the confidence intervals shown. 10,000 data sets of overlaps were randomly selected with replacement, and the calculations repeated on each one of them. The 10,000 results were sorted and used to provide the boundary results for the appropriate confidence intervals.

The distribution of disordered regions in the overlapping regions was compared to the overall distribution of disorder in the entire data set. The null hypothesis tested was that the distribution of disorder in overlapping regions is the same as that in the entire data set, that is, we assume that there is no bias toward a greater concentration of disordered residues in overlapping regions. Using a Chi-squared test on sequence positions located 15 residues apart (which satisfies the assumption of independence) we obtain a p-value that expresses the probability that our null hypothesis is correct.

Identification of putative ancestral, overprinted proteins

As a first screen, all proteins encoded by overlapping genes were subjected to SMART analysis (52) which includes prediction of PFAM and SMART domains, transmembrane and low complexity regions, signal peptides, etc. The sequences of all overlapping protein regions were analysed using 1) Psi-blast (2); 2) sequence profile comparison methods, which automatically run a Psi-blast query on a single sequence, align the retrieved sequence hits, derive a profile from the corresponding multiple sequence alignment, and search the library of sequence profiles PFAM –release 23- (25) for similar profiles: HHpred (86), Compass (74),

and FFAS03 (39); 3) fold recognition methods: Fugue (81) and Phyre (9). Finally, we submitted the 3D structures of proteins, when available, to structural similarity searches using VAST (30) and SSM (49). Protein regions were considered ancestral if they had statistically significant sequence or structural similarity with at least another protein region from a different viral family (unclassified genera were counted as distinct families).

Prediction of structural organization of pairs of known ancestral/novel overlapping regions

The above analyses (previous paragraph) identified known domains, transmembrane segments, etc. Refined disorder prediction was carried out as follows (respecting the principles described in (23)): we analyzed proteins containing novel or ancestral regions using the disorder predictor iPDA. For a conservative approach, we also used the predictors Prelink and Disopred, which have a very high specificity (113), when the presence of disorder in a certain region was dubious. If neither program predicted disorder within the region under scrutiny, we considered the whole region to be ordered. The boundaries of disordered regions were refined by visual inspection of hydrophobic cluster analysis (HCA) plots (14). To find experimental evidence of disorder, all proteins were subjected to a Blastp similarity search (2) against the database of disordered proteins Disprot (82), and we also carried out extensive bibliographical searches.

Analysis of amino acid composition

Composition Profiler (102) allows comparison of the composition of a user-defined "query" dataset (for instance overlapping regions of proteins) with that of another user-defined "background" dataset (for instance non-overlapping regions) or with that of a precompiled dataset. The precompiled datasets we used are SwissProt 51 (4), which is most similar to the distribution of amino acids in nature; PDB Select 25, which is a subset of structures from the Protein Data Bank (10) with less than 25% sequence identity, biased towards the composition of proteins amenable to crystallization studies; and DisProt 3.4 (82), which is a set of sequences of experimentally determined disordered regions. Composition profiler also allows the discovery of biases in certain groups of aa such as order-promoting aa or charged aa ("discover" option) (102), and the calculation of the relative entropy (RE) of two datasets, which roughly summarizes how dissimilar their composition is. We used a significance value of 0.01 to identify composition biases.

Disorder content of differentially constrained overlapping genes

The disorder content of viral overlapping genes whose evolutionary rates is known was calculated using the PONDR® VSL2 predictor. Protein sequences were taken from genome entries. The Genbank accession numbers of the genomes are: HBV: NC_003977; HTLV: AF139170; SIV: U72748; HPV: AF293961; ΦX174: J02482; PLRV: AF453389; Sendai: AB039658; CLCuV: NC_004607.

Results

Collection of a curated dataset of overlapping genes from a wide range of eukaryotic RNA viruses

We carefully selected overlapping genes whose existence was supported by experimental evidence. Indeed, including an overlapping reading frame that is in fact not translated might introduce noise in our analyses, since such sequences are not subject to evolutionary pressure. Misannotated overlaps might stem from untranslated "hypothetical" genes, or from a start codon wrongly assigned upstream of the true start codon, or from an undetected splicing event that results in an exon/intron overlap instead of an overlap of coding sequences. The latter possibility prompted us to exclude all viruses which are known to make use of splicing. Curation of prokaryotic viruses (bacteriophages) and of DNA viruses proved too difficult. Therefore, we focused on unspliced, eukaryotic RNA viruses, which are either single stranded with a plus or minus genome polarity (respectively +ssRNA and -ssRNA) or double stranded (dsRNA), and on unspliced retroid viruses, which use both DNA and RNA in their genome (for a review, see (5)). Only one representative virus per genus was chosen.

The construction and curation of the dataset is described in Material and Methods. We concentrated on overlaps longer than 90 nucleotides, corresponding to 30aa, for two reasons: i) shorter regions are unlikely to fold by themselves (87) and are thus expected to have a lesser structural impact, and ii) the reliability of disorder prediction increases with length (65, 90). By taking all of the above precautions, we built a very conservative, high quality dataset of 43 viral genomes containing *bona fide* overlapping genes.

Table 1 shows some statistics for the 43 viral genomes comprising our dataset, which are presented in Tables 2 to 6. They are grouped by taxonomy, to which we have paid particular attention in order to make this work as informative as possible (see Material and Methods).

Some viral genomes contain several pairs of overlapping genes (for instance the *arterivirus* GP2/GP3 and GP3/GP4 overlaps - Table 2), while some genes overlap with more than one gene —for instance the *orthohepadnavirus* P gene overlaps with 3 genes: L, X and the capsid gene (Table 3). Therefore, in total there are 52 gene overlaps (104 overlapping regions) in the dataset, involving 96 protein products (Table 1). All overlaps in the dataset are sense/sense, i.e. correspond to genes found on the same nucleic acid strand, and none encodes more than 2 proteins in different reading frames. The mean size of viral overlaps was 138 aa (Table 1), which corresponds to the typical size of a protein domain and is much longer than typical overlaps reported in bacterial genomes (29, 71). No precise data are available for eukaryotes due to the difficulty in reliably predicting overlapping genes, but a significant number of overlaps with a comparable length have been reported (1, 70).

Examples of *bona fide* overlapping genes that have not been incorporated in this study because of the above restrictions or because of technical limitations (see Material and Methods) include the *bornavirus* P/X gene overlap (109), removed because *bornaviruses* are known to make use of splicing (79), and the *henipavirus* P/V and P/C overlaps (106), excluded because the genome file contained a "join" instruction (see Material and Methods), generally indicative of splicing but in this case of a frameshift.

In spite of these limitations, our dataset still covers a wide evolutionary range. It mostly consists in ssRNA and dsRNA viruses, with only 2 retroid viruses (Table 3), because most retroid viruses are spliced and have thus been excluded. The dataset includes at least one representative from several large viral orders or supergroups: the (unofficial) alphavirus-like supergroup (72, 103) (Table 4), the orders *Picornavirales*, *Nidovirales* (Table 2), and *Mononegavirales* (Table 6), as well as the proposed order *Reovirales* (58) (Table 2). Thus, our dataset represents a good sampling of the diversity of overlapping genes in RNA viruses.

Proteins regions encoded by overlaps have a higher disorder content

We have chosen to use the PONDR® VSL2 software for the automated analysis because it has consistently been found to have one of the best combinations of specificity and sensitivity (88) and because its definition of "disorder" is well suited to the biological question studied. Indeed, when PONDR VSL2 predicts a region as "disordered", what it predicts, more precisely, is that it has no fixed 3D structure (69), which corresponds to our hypotheses about overlapping gene products (see introduction). In addition to PONDR, we also carried out in-depth analysis on selected proteins using a combination of structural prediction methods, as described in Material and Methods and below. Our strategy is described in Fig. 2.

All proteins encoded by overlapping genes were subject to prediction of structural disorder using PONDR® VSL2. As shown in Fig. 3, 29% of the aa of the whole dataset are predicted to be in a disordered state. This is distributed in relation to overlapping as follows: 23% of the aa in non-overlapping regions are predicted as disordered, to be compared with 48% of the aa in overlapping regions. This difference in disorder content is highly significant (chi-square = 254.4, one degree of freedom, P-value = 2.7×10^{-57}, see Material and Methods). Thus, in our dataset, protein regions encoded by overlapping genes show a significant bias towards structural disorder.

Identification of ancestral/novel protein pairs by their phylogenetic distribution

One of our hypotheses (see introduction) was that novel proteins created by overprinting tend to be disordered. Therefore, we tried to identify overlaps encoding recognizable ancestral/novel protein pairs.

Finding which protein is the ancestral one and which is the novel one in an overlapping pair is a difficult problem. Methods include 1) comparison of the codon usage of each overlapping reading frame to that of non-overlapping genes of the viral genome (67, 68) and 2) assessing the phylogenetic distribution of each overlapping gene product, i.e. the extent to which they have homologs in other organisms (43, 71). In these

methods, the ancestral reading frame is supposed to be respectively the one having the standard genome codon usage or the one with the widest phylogenetic distribution. Whenever possible, both methods should be used together since they are complementary (43). However, implementing the first method on nearly 100 viral proteins is a large project in itself and is clearly out of the scope of this work. Therefore, we chose to examine the phylogenetic distribution of each overlapping gene product. We presumed that a protein region (>30aa) involved in an overlap was ancestral only if it was **conserved in at least 2 viral families**. Given the fast rate of evolution of RNA viruses (20), this is a very stringent, and thus very conservative, criterion.

Our strategy is described in Fig. 2 and in Material and Methods. Briefly, protein regions were considered ancestral only if they had either statistically significant sequence similarity or structural similarity with at least another protein region from a *different* viral family. Sequence similarity was assessed using profile-profile comparison and structural similarity was assessed using fold recognition methods or direct structural comparison.

We found 21 protein regions matching this criterion, coming from 20 proteins from 19 viral genera. They are presented in Table 7. Several viral families contain genera with homologous pairs of overlapping genes (i.e. both overlapping regions have homologs in another viral genus, which also overlap): the *Birnaviridae* VP5/VP2 overlap; the *Tubiviridae* TGB2/TGB3 overlap; the *Tombusviridae* movement protein (MP) / p19 or p14 overlap (Table 7). In these cases we retained only one viral genus per family (respectively *Avibirnavirus*, *Pomovirus* and *Tombusvirus*). In the end we found 17 non-homologous overlaps encoding ancestral regions, from 15 different genera corresponding to 9 families of +ssRNA, dsRNA, and retroid viruses (Table 7).

All ancestral regions match at least one PFAM sequence family as shown using profile-profile comparison (see Material and Methods); in other terms, no ancestral region was selected only on the basis of structural similarity. (Briefly, a PFAM family is a collection of sequences of homologous protein domains or regions (25). Related PFAM families are grouped in "clans" (24)).

We found no gene overlap for which both protein products were presumed ancestral according to the phylogenetic distribution criterion. In other terms, all the overlaps selected by this method encoded, on the one hand, a protein region conserved in at least 2 viral families and on the other hand, a protein region that was restricted to one family at most. This reinforces our working hypothesis that protein regions conserved in 2 viral families can be considered ancestral whereas the regions overlapping them are novel (see also the Discussion). Table 7 presents novel protein regions together with the ancestral protein regions that they overlap.

Some ancestral regions have homologs in a very large number of viral families and it would be highly impractical to mention all these viral families. Instead, we present in Table 7 the PFAM families (release 23) corresponding to ancestral regions. This allows the reader to visualize easily the taxonomic distribution of homologs of ancestral regions thanks to a user-friendly service called "species", available on the PFAM web site as well as relevant bibliographical references (25).

During the analysis of this large dataset, we uncovered evolutionary relationships between some viral proteins, using profile-profile comparisons (see Material and Methods). In Table 7 we propose corresponding new PFAM families and clans (24). Two of these suggested clans correspond to distant sequence similarities unreported so far, to our knowledge. The first involves the nucleoproteins of the *Bunyaviridae* and of the unclassified genus *tenuivirus*. The second involves the C-terminal moiety of the methyltransferase-guanylyltransferase (MT-GT) (72) of the Altovirus group, called "Y region" (45). We found that it is also present in the Typovirus group and is thus conserved throughout the alphavirus-like supergroup (Table 4). This finding is coherent with experimental evidence that the MT-GTs of this viral supergroup have a common mechanism (56). This MT-GT is unique to these viruses, and thus constitute an important drug target for a number of human pathogens like HEV or chikungunya virus. Its structure has not been solved at present, and thus our finding might facilitate further protein expression studies or modelling studies.

Prediction of the structural organization of ancestral and of novel proteins

We then predicted the structural organization of each ancestral and novel protein using a combination of complementary methods (see Material and Methods and Fig. 2) and plotted it in Fig. 4. All 17 ancestral protein regions (Fig. 4, in dark grey) were predicted as ordered (wide boxes). Out of the 17 novel protein regions (Fig. 4, in light grey), 6 are predicted as mostly ordered (wide boxes): *carmovirus* p25, *tombusvirus* p19, *orthohepadnavirus* S domain, *capillovirus* replicase, *orthobunyavirus* NSs, and *carmovirus* p23; 1 is predicted as about half ordered: the *potexvirus* TGBp3, and the 10 others are predicted as mostly disordered (narrow boxes). Thus, these results suggest a greater tendency for intrinsic disorder in novel protein regions, which is compatible with the first hypothesis emitted in the introduction.

Biased sequence composition of protein regions encoded by overlaps

Earlier studies have suggested that overlapping protein regions have a biased sequence composition, being enriched in aa with the highest codon degeneracy, *ie* each encoded by 6 different codons (68). We performed an exploratory analysis based on our larger dataset. Using Composition Profiler (102), we first examined global biases in aa composition, represented by the “relative entropy” (see below), then biases in specific aa. We compared the sequence composition of all overlapping regions, or of novel or ancestral regions (Table 7

and Fig. 4), to that of reference sets: Swiss-Prot, PDB and Disprot. Roughly, they correspond respectively to the mean composition of proteins in nature, to that of ordered proteins and to that of disordered proteins (see Material and Methods). To examine biases in global composition, we calculated the Relative Entropy (RE) between each dataset and Swiss-Prot, which is a rough measure of their difference in mean composition (102) (see Material and Methods). The higher the RE of two datasets, the more they differ in composition. For instance, the RE of PDB and of Disprot relative to Swiss-Prot are respectively 0.002 and 0.07 (Fig. 5), which indicates that Swiss-Prot has a composition much closer to that of PDB than to that of Disprot.

Fig. 5 clearly shows that overlapping regions (4th bar from the left) have an important composition bias relative to Swiss-Prot (RE lower than that of Disprot but much higher than that of PDB). Considering the subset of ancestral/novel regions (listed in Table 7), we see that ancestral regions have an RE only slightly lower than that of all overlapping regions (compare 5th and 4th bar), but that novel regions (6th bar) have a spectacular composition bias, with an RE more than twice that of Disprot. As a control, the RE of the "background" composition is much lower than that of the overlapping datasets (compare bar 3 and bars 4 to 6).

Then, we computed the relative enrichment or depletion *in specific aa* of our datasets with respect either to Swiss-Prot or to non-overlapping regions (used as a “background” composition of viral proteins). The biases uncovered when comparing the datasets to the background were similar to those observed when compared to Swiss-Prot but of lower magnitude (not shown). Consequently, in order to draw conservative conclusions, we present the composition bias of each aa relative to this background instead of Swiss-Prot, in Fig. 6. Aa are arranged according to their codon degeneracy as in (68), indicated at the bottom of the figure. We also examined whether the datasets were significantly ($P<0.01$) biased in disorder-promoting or in order-promoting aa (listed in (102)) using the "Discovery" option of Composition Profiler (see Material and Methods) and indicated it on the right part of Fig. 6.

Taken together, overlapping regions have a significant deviation in most aa (16 out of 20), and are significantly biased towards disorder, *i.e.* enriched in disorder-promoting aa and depleted in order-promoting aa (Fig. 6, top panel). The subsets of ancestral and of novel regions show distinct trends. Ancestral regions have a composition bias for 3 aa only (second panel) and have no significant bias towards order or disorder. On the contrary, novel regions (third panel) are heavily biased regarding both the number of aa involved (18) and the magnitude of the bias (on average more than twice that of overlapping regions taken globally, compare top and third panel). Furthermore, they are biased towards disorder (lower panel, right).

Finally, we examined Fig. 6 qualitatively, looking for a bias of overlapping regions with respect to codon degeneracy: for instance enrichment in aa encoded by highly degenerate codons (as reported in (68)), or

depletion in aa encoded by low-degeneracy codons. This simple visual examination suggests that overlapping regions taken globally (top panel) are enriched in aa with a codon degeneracy ≥ 4 and depleted in aa with a degeneracy <4. However, the magnitude of this bias depends upon the dataset chosen as background (Swiss-Prot or non-overlapping regions, not shown) and it should be taken with great care until validated by a rigorous statistical analysis on a larger dataset. No clear bias with respect to codon degeneracy is visible for either the novel or ancestral regions (Fig. 6, middle and lower panel).

In summary, the composition of overlapping protein regions is biased towards disorder-promoting aa. In particular, novel regions have a very large compositional bias. Overlapping regions seem to favour the use of aa with a high (≥ 4) codon degeneracy, as seen using a merely qualitative approach, but this observation should be taken with caution until validated by further studies.

Specific functions of overlapping proteins

In Table 7, we have compiled the known functions of overlapping proteins. In most cases one or several function(s) have been ascribed to the full-length protein but the precise function of the novel region itself has not been determined. In cases where a function has been ascribed specifically to the novel region, we included it with the associated bibliographical references. Table 7 and Fig. 4 show that all novel overprinting proteins with known function, except one (the *orthohepadnavirus* L), are "accessory" proteins (i.e. neither structural nor enzymatic), most often overprinting a structural or enzymatic protein.

Proteins generated by overprinting homologous DNA sequences are extremely diverse

Several ancestral viral proteins of our dataset, from different genera, are homologous to each other (*i.e.* they share statistically significant sequence similarity). They have been overprinted by proteins which show no distinguishable sequence or structural similarity to each other and thus might have been created independently in each genus. The identification of such proteins, which show a wide diversity both in function and in structure, offers an unprecedented insight in *de novo* protein creation by viruses. For instance, consider Fig. 4, panel 4, and the corresponding Table 7. *Capilloviruses, tombusviruses* and *umbraviruses* encode a movement protein belonging to the "30K" superfamily, sharing a homologous central domain (61). In these genera, the movement protein has been overprinted respectively by an ordered domain of unknown function that is part of a polyprotein, by a mostly ordered suppressor of RNA silencing (105), and by a ribonucleoprotein (which also plays a role in long distance movement) that is predicted disordered but might undergo a disorder to order transition upon binding to RNA (92). The case of *mandariviruses*, *trichoviruses*, and *capilloviruses* (same panel) which all encode a homologous coat protein (18, 44), is as striking. In the first two genera, it has been overprinted respectively by the disordered N-

terminal domain of an RNA-binding protein and by the disordered C-terminal domain of a 30K movement protein, while in *capilloviruses* it is not part of an overlap.

Finally, Fig. 4, panel 3, shows that regions homologous to the Shell (S) domain of the superfamilies of capsids having the SCOP fold "Nucleoplasmin-like/VP (viral coat and capsid proteins)" (3) have been overprinted in several taxonomically distant viruses by very diverse protein regions: the *avibirnavirus* VP5, a disordered anti-apoptosis protein (36); a disordered tail of the *betatetravirus* replicase; a disordered tail of *machlomovirus* p31; and a region of the *carmovirus* p25 that contains a predicted transmembrane segment (the last three having an unknown function). These examples highlight the "creativity" of nature, which, although starting from a similar material (homologous DNA sequences) did not "invent" similar proteins twice.

Disorder and sequence constraints on overlapping reading frames

Several studies have shown that overlapping genes often encode a protein heavily constrained in sequence and another one that is much less constrained (28, 32, 37, 59, 63, 64, 67, 77, 98). In these cases, we would expect the protein with the less constrained sequence to have the greater disorder content, since disordered proteins are less sensitive to sequence changes.

Measuring sequence constraints of overlapping reading frames is usually done by comparing the rate of synonymous substitutions to that of non-synonymous substitutions for each frame, using closely related genome sequences; the frame for which this ratio is higher is considered the most constrained (38, 71). Performing such analyses on our entire dataset was beyond the scope of this work, so, in order to provide some verification to the above hypothesis we gathered from the literature all studies that provide information on the evolutionary rate differences between specific sets of viral overlapping genes (28, 32, 37, 59, 63, 64, 67, 77, 98). For each, we performed disorder predictions on the corresponding protein products using PONDR® VSL2.

Fig. 7 plots the predicted disorder content of both regions encoded by each overlap. It clearly shows that, in 8 cases out of 10, the less constrained frame encodes the protein region with the greatest disorder content. In another case, that of human papillomavirus (HPV), the less constrained protein (E2) is only marginally less disordered than the more constrained (E4): 89% vs 100% respectively, which in fact corresponds to both proteins being almost entirely disordered. The last overlap (ΦX174) corresponds to regions of proteins D and E predicted to be both ordered. Thus, this preliminary exploration supports the idea that the less constrained reading frame generally encodes the most disordered region. However, this is not an absolute rule and overlapping frames can encode two ordered protein regions simultaneously (such ordered/ordered overlaps can also be found in our dataset, see Fig. 4).

Discussion

Our carefully curated dataset and conservative analysis allow us to make a strong case for our prediction that proteins encoded by gene overlaps tend to be disordered, and to offer unprecedented insight in their evolution.

Unfortunately, it was difficult to find experimental evidence relating to our predictions of disorder, in part because many proteins considered herein are accessory ones, which are poorly characterized (see below). Examples of disorder predictions experimentally confirmed include the *orthohepadnavirus* protein X (73); the N-terminal "arm" of the capsid proteins of *omegatetraviruses* (35) (Fig. 4) and *sobemoviruses* (51); the N-terminal moiety of the P proteins of *morbilliviruses* (42) and *vesiculoviruses* (17). We could not find any evidence in the literature that would contradict our predictions, even though some regions predicted as disordered can actually become partially ordered, e.g. the basic, N-terminal "arm" of the capsid proteins of a number of icosahedral viruses (51). However, this corresponds to the definition of disorder used in this work (see introduction): proteins do not have a unique, rigid 3D structure.

Regarding our prediction of ancestral protein regions (Fig. 4), there is good evidence for most that they are correct. For instance, the RT of *orthohepadnaviruses* belongs to an ancient enzyme family (83); likewise, the S domain of capsid proteins (34), the 30K domain of movement proteins (61), and the MT of the alphavirus-like supergroup (72) are each found in more than a dozen virus families. Furthermore, evolutionary studies on viruses of our dataset that used complementary analyses, such as codon usage, are in agreement with our results: they predict that the *tymovirus* polyprotein (68) and the *birnavirus* VP2 are ancestral (93).

We hope to obtain further insights from other organisms. For instance, we noticed a few exciting examples of ancient proteins overprinted by proteins predicted or known to be disordered (mentioned between brackets): the ankyrin domain of mammalian $p16^{INK4}$ ($p19^{ARF}$) (15) and the bacterial ribosomal protein L34 (N-terminal extension of RNAse P) (22).

Earlier observations on the properties of proteins encoded by overlapping genes

There have been earlier anecdotal observations of a connection between gene overlap and structural disorder. Jordan & al suggested that the emergence of protein C in the P/C overlap of *Paramyxoviridae* (see Table 6) was favoured by the disordered nature of P (40). Likewise, Narechania *& al* noticed that a disordered region of the *Papillomaviridae* protein E2 might have favoured the overprinting of protein E4, also predicted to be disordered (64). However, these studies gave no reliable evidence that P and E2 were ancestral.

More recently, Meier & al expressed ideas similar to this work, based on the analysis of a single overlap (60). They suggested that the abundant disorder observed in the crystal structure of the *coronavirus* protein

NSP9, most likely created by overprinting the nucleoprotein (N), may reflect its recent creation as well as constraints imposed by the N reading frame.

Prior to this article, there had been only one systematic study of overlapping genes at the protein level (68). It reported that proteins encoded by overlaps were enriched in aa with the highest codon degeneracy (R, L and S). We found enrichment in R and S but not in L and no clear-cut influence of codon degeneracy. The difference might be due to the much lower number of viral genera sampled in the previous work (68).

Recent work on (uncurated) protein products of overlapping genes of RNA viruses has made interesting connections between their relative frames, their age and the mode of creation of the overlap (8). Our dataset of ancestral/novel protein regions is too small to reliably analyze their findings, but we plan to do so once a larger dataset is created.

Why structural disorder in protein products of overlapping genes?

In the introduction, we proposed 2 non-exclusive hypotheses to explain the increased occurrence of disorder in proteins encoded by gene overlaps: either 1) the newborn protein in each pair tends to be disordered, or 2) the presence of disorder in either protein encoded by overlapping genes lessens evolutionary constraints. In fact, our results are compatible with both hypotheses.

Indeed, almost two thirds of novel, overlapping protein regions are disordered (Fig. 4), to be compared with less than one fourth of non-overlapping protein regions (Fig. 3), which is compatible with hypothesis 1). However, these results should be validated by further studies since we could only determine novel/ancestral status for 21 overlaps out of 52.

The analysis summarized by Fig. 7 is also compatible with hypothesis 2). A number of studies have shown that overlapping genes most often encode one heavily constrained protein and another one much less constrained (28, 32, 37, 59, 63, 64, 67, 77, 98). Our analysis of a limited dataset formed with the proteins studied in these works suggests that the less constrained proteins are generally the more disordered, which is consistent with hypothesis 2).

Thus, it is possible that both factors invoked in hypotheses 1) and 2) actually contribute to the increased disorder content of overlapping gene products. A simple and attractive explanation would be that the novel proteins of each pair generally are the less constrained ones. Further studies will be needed to address this question.

Insights for viral bioinformatics

This work establishes several methodological points:

It is possible, with a reasonable effort, to make a thorough bioinformatics structural analysis on a large number (~100) of proteins involved in a given biological question. At present, this kind of analysis is quite rare (e.g. (31)) although it obviously adds great value when compared to global statistics (e.g. compare Fig.

3 and 4). Furthermore, such analyses are feasible by bench virologists, thanks to the availability of user friendly web-based tools such as the MPI toolkit (11).

Our work also suggests that viral ORFs overlapping a known coding sequence and encoding hypothetical proteins with highly biased sequence composition, which are often considered non-coding (99) and discarded, might in fact encode a protein. Indeed, recent exciting discoveries of overlapping genes using a systematic approach (26) suggest that overlapping genes in viruses might be even more common than previously thought.

Most studies aimed at determining the ancestral protein encoded by a gene overlap did not take into account domain organization, with a few exceptions (28, 64, 67). However, the present work makes it clear that overlapping gene products are often composed of several domains that might have different evolutionary histories. For instance, the overlapping parts of the *capillovirus* replicase and movement protein are each composed of several domains, are is the overlapping part of the *tymovirus* replicase (Fig. 4). Thus, analyses of overlapping gene evolution should be carried out by studying domains separately.

The study of *de novo* proteins should enhance our knowledge of protein space

At present, it is thought that proteins adopt less than 10,000 structural folds in nature, much less than expected from our understanding of biophysics (115). This discrepancy has brought about two main hypotheses: 1) some structural folds are favoured by nature, for unknown biophysical or functional reasons; 2) most proteins are descended from a limited set of ancestors by duplication (for a review, (116)).

All solved structures of overprinting proteins presented here and elsewhere correspond to previously unobserved folds (53, 60). This constitutes a challenge to hypothesis 1) above and even suggests that we might underestimate the number of folds created in nature, because of our limited knowledge of the 3D structures of *de novo* proteins. Solving them (as advocated by Gibbs and Keese, remarkably, more than 15 years ago (43)) might thus help to improve methods to predict the 3D structure of proteins from their sequence, a central problem of bioinformatics which crucially depends on knowing the diversity of protein folds (33).

De novo protein creation, a significant factor in evolution?

We noted in the Results that the great majority of novel proteins are "accessory" (i.e. neither structural nor enzymatic), most often overprinting a structural or enzymatic protein, confirming an earlier observation (8). "Accessory" does not mean that they are dispensable *in vivo*; on the contrary, most novel regions play an important role in viral pathogenicity or spread (Table 7), as noticed by Li and Ding (53). Thus, *de novo* protein creation appears to be a significant factor in viral evolution, in particular in the evolution of pathogenicity, poorly understood at present.

Is it limited to overprinting by viruses? At the time of submitting this article, 2 systematic studies on *de novo* protein creation in eukaryotes (from non-coding sequences, and thus not generating overlapping genes) were published. They indicate that *de novo* protein creation occurs at a significant and unexpected rate, having generated between 5% and 20% of orphan proteins of primates (95), and about 12% of orphan proteins of the genus *Drosophila* (118). Reciprocally, almost all *de novo* viral proteins we identified are orphans at the genus level, i.e. are restricted to one genus at most (see Table 7). Thus, these works and ours provide numerous examples of orphan proteins created *de novo*, as opposed to having diverged beyond recognition from other relatives (see introduction).

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Author contributions

C.R Gathered and classified all complete, unspliced RNA viral genomes and extracted the overlapping genes.
M.K. Performed the order-disorder prediction and initial analysis of the genomic dataset
K.D. Coordinated the disorder prediction study
P.R. Supervised the disorder prediction study and performed statistical analysis on the genomic dataset. Gathered the data and analyzed the relationship between evolutionary constraints and intrinsic disorder. Co-wrote the manuscript
D.K. Conceived and coordinated the study, curated the overlapping gene dataset, performed the remaining bioinformatics analyses, and co-wrote the manuscript.
All authors read and accepted the final manuscript.

Figure captions

Fig. 1 Creation of a novel protein region by overprinting

Top: a DNA sequence encodes 2 proteins in different reading frames. Notice the potential, unused stop codon downstream of protein X. **Middle**: a mutation abolishes the stop codon of protein X, causing its elongation ("overprinting") until the pre-existing stop codon. This results in a gene overlap. **Bottom**: the overlap encodes an overprinted (ancestral) protein region, in dark grey, and an overprinting (novel) protein region, in light grey.

Fig. 2 structural and functional prediction workflow: ex. of the *betatetravirus* replicase/capsid overlap

Conventions are the same as in Fig. 1. Second panel: superimposed PONDR prediction for the capsid (dark grey) and replicase (light grey). Regions with a score above 0.5 are predicted disordered. Third panel, predictions of the boundaries of ancestral and novel regions of the replicase and capsid (see text). Bottom: result of refined structural and functional analysis (see text). Wide and narrow boxes correspond respectively to predicted order and disorder. Domain names were obtained from the literature. Note the good agreement between automated PONDR predictions and the refined analysis.

Fig. 3: Predicted disorder content of proteins encoded by overlapping genes

The prediction was made using PONDR VSL2. The error bars correspond to a 95% confidence interval.

Fig. 4: structural and functional organisation of recognizable ancestral/novel overlapping protein regions

Proteins encoded by overlapping genes are represented to scale with the same conventions as in Fig. 1 and 2. Boundaries of ancestral and novel regions are given in Table 7.

Each panel represents different cases of overprinting. For instance, the third panel represents all novel proteins that have overprinted homologous capsid proteins. The name of the panel refers to the PFAM family (in brackets) or clan (in square brackets), actual or proposed herein, to which ancestral protein regions belong (see text and Table 7). Ancestral regions within a given clan are aligned vertically (eg the 30K domain of *umbra-, tombus-, and capilloviruses* movement proteins, panel 4).

Beware: domains bearing a similar name are not always homologous. For instance in panel 2 the *pomovirus* and *potexvirus* TGBp2 are homologous (they belong to the family Plant_vir_prot) whereas the *pomovirus* and *potexvirus* TGBp3 are not (they belong respectively to the β C/D and 7K families, see Table 7). Likewise, there is no evidence that the RNA-binding "arms" of capsid proteins of different genera are homologous (panel 3).

Abbreviations: 30K, conserved domain of the 30K family of movement proteins; al: antigenic loop; B (or B1 or B2): base domain (or subdomain); Flexi coat, central conserved region of flexuous viral coats; Ig, Immunoglobulin-like domain; L, large envelope protein; LDM, long-distance movement protein; NABP, nucleic-acid binding protein; MT-GT: methyltransferase-guanylyltransferase; Prol-rich, proline-rich region; RNP, ribonucleoprotein; Rdrp: RNA-dependent RNA polymerase; RT, reverse transcriptase; S (or S1 or S2): shell domain (or subdomain); tm: transmembrane segment; TGBp2 and TGBp3: triple gene block protein 2 and 3; TP, terminal protein.

Fig. 5 Relative entropies (RE) of overlapping or non-overlapping protein regions versus Swiss-Prot

The Relative Entropy (RE) of two datasets is a rough measure of their difference in mean aa composition (see text). We have plotted, from left to right: as a standard, the RE of biologically meaningful datasets (PDB, Disprot) with respect to Swiss-Prot; the RE of non-overlapping regions (representative of viral proteins) with respect to Swiss-Prot; and the RE with respect to Swiss-Prot of either all overlapping regions, of ancestral regions, or of novel regions.

Note that ancestral and novel regions form only a subset of all overlapping regions since for some pairs of overlapping regions we could not determine which was the ancestral one and which was the novel one.

Fig. 6 Deviation in sequence composition of overlapping protein regions relative to the background composition of non-overlapping regions

Relative enrichment (positive values) or depletion (negative values) in aa of each dataset with respect to that non-overlapping regions, in % (see text). For easier visualization, we have plotted values only for the aa that show a statistically significant bias ($P<0.01$).

Aa are arranged according to their level of codon degeneracy, indicated below the lower panel (a codon degeneracy of 3 for Isoleucine (I) means that 3 codons code for Isoleucine). The dashed vertical lines separate aa with a high codon degeneracy (≥ 4) from those with a low degeneracy (≤ 3).

Note that the datasets of novel and ancestral regions (2280 aa each) represent only 22% of the aa contained in "all overlapping regions". Thus the composition of all overlapping regions is not expected to correspond exactly to the mean composition of the ancestral and novel subsets.

Figure 7. Evolutionary constraints of overlapping protein regions and their disorder content

Predicted disorder content is plotted for overlapping protein pairs from several viruses, listed below the graph. In each pair, the first protein listed is the more constrained. Bars indicate the percentage of disorder in the overlapping part of these proteins.

Abbreviations: HBV: Hepatitis B virus; CLCuV, Cotton leaf curl virus; SIV: Simian immunodeficiency virus; HTLV: Human T-lymphotropic virus; ΦX174: coli-phage ΦX174; PLRV: Potato leafroll virus; HPV: Human papillomavirus.

Tables

Table 1. Properties of the overlapping gene dataset

Type of nucleic acid	Number of families[1]	Number of genera[1]	Number of overlapping gene pairs[2]	Number of proteins affected by overlaps[3]
+ssRNA	13	27	30	58
-ssRNA	4	8	12	20
dsRNA	3	6	6	12
Retroid	2	2	4	6
All viruses	**22**	**43**	**52**	**96**
Total number of residues			42 656 aa	
Number of residues encoded by overlaps			16 175 aa (38%)	
Length of protein region encoded by overlap		Min.	36 aa (arterivirus)	
		Max.	626 aa (tymovirus)	
		Mean	**138 aa**	

Repartition of collected viruses by taxonomy, and various statistics.

(1): distinct, unassigned genera or unassigned families are counted as *bona fide* genera or families

(2): some genera contain several overlapping gene pairs

(3): some genes overlap with more than one gene

Table 2. overlapping genes in unspliced viruses of the orders *Reovirales, Picornavirales and Nidovirales*

Order (*-virales*)	Family (*-viridae*)	Genus (*-virus*)	Virus species	Genome accession number	Protein products	Protein accession number	Boundaries of overlap (aa)	
							Start	End
Proposed order *Reovi*	*Birna*	*Aquabirna*	*Infectious pancreatic necrosis*	NC_001915	VP5	NP_047195	3	133
					VP2 (capsid)	NP_047196	1	131
		Avibirna	*Infectious bursal disease*	NC_004178	VP5	NP_690837	16	149
					VP2 (capsid)	NP_690838	1	134
	Reo	*Orthoreo*	*Mammalian orthoreo 1*	NC_004267	sigma-1a (hemaglutinin)	NP_694621	21	139
					sigma-1bNS	NP_694622	1	119
		Oryza	*Rice ragged stunt*	NC_003771	Replicase	NP_620541	160	485
					P4b	NP_620542	1	326
		Phytoreo	*Rice dwarf*	NC_003768	Pns12	NP_620538	91	182
					OP-ORF	(a)	1	92
	Toti	*Toti*	*Saccharomyces cerevisiae L-BC (La)*	NC_001641	Capsid (Gag)	NP_042580	649	697
					Replicase (Pol)	NP_042581	1	49
Picorna	*Picorna*	*Cardio*	*Theiler's*	NC_001366	L (polyprotein, VP4)	NP_040350	5	160
					L* ("L star").	(a)	1	156
Nidovi	*Arteri*	*Arteri*	*Lactate dehydrogenase-elevating*	NC_001639	GP2	NP_042574	184	227
					GP3	NP_042575	1	44
					GP3	NP_042575	156	191
					GP4	NP_042576	1	36

Details of overlapping genes and the proteins they encode. To avoid unnecessary cluttering, the taxon endings have been shortened (see top row). For instance the third row should read: order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus*, species *Lactate dehydrogenase-elevating virus*. Common alternative names of proteins are given between brackets. A comprehensive list of alternative names of viral proteins can be found in the database Virgen (http://bioinfo.ernet.in/virgen/virgen.html) (50).

Abbreviations: GP, glycoprotein; L, large protein.

(a) Several proteins are not mentioned in the NCBI genome file and thus have no accession numbers, although their existence has been proven (see Material and Methods). We provide their sequence in File S1.

Table 3: overlapping genes in unspliced retroid viruses

Family (-viridae)	Genus (-virus)	Virus species	Genome accession number	Protein products	Protein accession number	Start of overlap (aa)	End of overlap (aa)
Caulimo	*Badna*	*Cacao swollen shoot*	NC_001574	Polyprotein	NP_041734	1721	1834
				ORF5	NP_041736	1	114
Hepadna	*Orthohepadna*	*Arctic ground squirrel hepatitis B*	NC_001719	Capsid precursor (E antigen precursor)	NP_043862	166	217
				P (pol)	NP_043864	1	52
				P (pol)	NP_043864	188	614
				L	NP_043865	1	427
				P (pol)	NP_043864	793	877
				X protein	NP_043868	1	85

Conventions are the same as in Table 2. Abbreviations: L, large envelope protein; P, polymerase.

Table 4: overlapping genes in +ssRNA viruses of the alphavirus-like supergroup

Group or order	Family (*-viridae*)	Genus (*-virus*)	Virus species	Genome accession number	Protein products	Protein Accession number	Boundaries of overlap (aa) Start	End
Group Altovirus[1]	*Bromo*	*Cucumo*	*Cucumber mosaic*	NC_002035	Replicase	NP_049324	778	857
					2b	NP_619631	1	80
		Ilar	*Spinach latent*	NC_003809	Replicase	NP_620678	696	797
					2b	NP_620679	1	102
	Hepe	*Hepe*	*Hepatitis E*	NC_001434	Capsid protein (ORF 2)	NP_056787	1	110
					ORF3 (P)	NP_056788	14	123
	proposed family *Tubi*	*Hordei*	*Barley stripe mosaic*	NC_003481	TGBp2 (beta C)	NP_604488	69	131
					TGBp3 (beta D)	NP_604489	1	63
		Peclu	*Indian peanut clump*	NC_004730	P14 (TGBp2)	NP_835266	71	122
					P17 (TGBp3)	NP_835267	1	52
		Pomo	*Potato mop-top*	NC_003725	TGBp2	NP_620439	72	119
					TGBp3	NP_620440	1	48
Group Typovirus[1] (proposed order *Tymovirales)*	*Tymo*	*Tymo*	*Turnip yellow mosaic*	NC_004063	Movement protein (OP)	NP_663296	3	628
					Replicase	NP_663297	1	626
	Flexi	*Capillo*	*Apple stem grooving*	NC_001749	Polyprotein	NP_044335	1584	1903
					Movement protein (36K)	NP_044336	1	320
		Carla	*Blueberry scorch*	NC_003499	Coat protein	NP_612812	268	312
					NABP (16kD)	NP_612813	1	45
		Tricho	*Apple chlorotic leaf spot*	NC_001409	Movement protein	NP_040552	356	460
					Coat protein	NP_040553	1	105
		Mandari	*Indian citrus ringspot*	NC_003093	Coat protein	NP_203557	226	325
					NABP (23kD)	NP_203558	1	100
		Potex	*Cassava common mosaic*	NC_001658	TGBp2 (movement protein)	NP_042697	63	112
					TGBp3	NP_042698	1	50

Conventions are the same as in Table 2. Abbreviations: NABP, nucleic acid-binding protein; OP, overlapping protein; P, phosphoprotein; TGBp, protein encoded by the triple gene block. (1) Unofficial taxons (see first paragraph of Results).

Table 5: overlapping genes of +ssRNA viruses which do not belong to any order or supergroup

Family (-viridae)	Genus (-virus)	Virus species	Genome accession number	Protein products	Protein accession number	Boundaries of overlap (aa)	
						Start	End
Barna	*Barna*	*Mushroom bacilliform*	NC_001633	ORF1	NP_042508	3	179
				Vpg-protease	NP_042509	1	177
				Vpg-protease	NP_042509	605	657
				Replicase	NP_042510	1	53
uncl.	*Sobemo*	*Sesbania mosai*	NC_002568	Polyprotein	NP_066392	900	962
				capsid	NP_066394	1	63
Noda	*Alpha-noda*	*Flock house*	NC_004146	A (replicase)	NP_689444	900	998
				B2	NP_689446	1	99
	Beta-noda	*Striped Jack nervous necrosis*	NC_003448	protein A (replicase)	NP_599247	893	967
				B (B2)	NP_599248	1	75
	Nd	*Macro-brachium rosenbergii noda*	NC_005094	Replicase	NP_919036	901	1033
				B2	NP_919037	1	133
Tetra	*Betatetra*	*Nudaurelia capensis beta*	NC_001990	Replicase	NP_048059	1316	1925
				Coat	NP_048060	1	610
	Omegatetra	*Dendrolimus punctatus tetra*	NC_005899	p17	YP_025095	32	158
				p71 (capsid)	YP_025096	1	127
uncl.	*Umbra*	*Tobacco bushy top*	NC_004366	RNP (LDM)	NP_733849	6	237
				MP	NP_733850	1	232
Tombus	*Aureus*	*Pothos latent*	NC_000939	Movement protein (27K)	NP_051033	44	173
				14K	NP_051034	1	130
	Carmo	*Hibiscus chlorotic ringspot*	NC_003608	Replicase (p28/p81)	NP_619671	4	212
				p23	NP_619673	1	209
				Coat	NP_619676	5	228
				p25	NP_619677	1	224
	Machlomo	*Maize chlorotic mottle*	NC_003627	p31	NP_619720	130	279
				Coat	NP_619722	1	150
	Necro	*Tobacco necrosis D*	NC_003487	$P7_1$	NP_608313	13	62
				P7a	NP_608314	1	50
	Tombus	*Cymbidium ringspot*	NC_003532	MP	NP_613263	11	182
				p19	NP_613264	1	172

Table 6: overlapping genes in unspliced –ssRNA viruses

Order (*-virales*)	Family (*-viridae*)	Genus (*-virus*)	Virus Species	Genome accession number	Protein product	Protein accession number	Boundaries of overlap (aa) Start	End
-	*Bunya*	*Orthobunya*	*Bunyamwera*	NC_001927	N	NP_047213	7	107
					NSs	NP_047214	1	101
Mononega	*Paramyxo*	*Morbilli*	*Measles*	NC_001498	P	NP_056919	232	299
					V	(a)	232	299
		uncl.	*Tupaia paramyxo*	NC_002199	P	NP_056919	8	193
					C	NP_056920	1	186
					P	NP_054691	230	282
					V	NP_054692	230	282
					P	NP_054691	9	161
					C	NP_054693	1	153
		uncl.	*Mossman*	NC_005339	P	NP_958049	244	295
					V	NP_958050	244	295
					P	NP_958049	11	162
					C	NP_958051	1	152
		Respiro	*Sendai*	NC_001552	C'	NP_056872	8	215
					P	NP_056873	1	208
					P	NP_056873	318	369
					V	(a)	318	369
		Rubula	*Mumps*	NC_002200	P	NP_054708	156	224
					V	NP_054709	1	224
	Rhabdo	*Vesiculo*	*Vesicular stomatitis Indiana*	NC_001560	NS	NP_041713	25	91
					C'	(a)	1	67
	Filo	*Ebolavirus*	*Reston Ebola*	NC_004161	SGP	NP_690583.1	297	367
					sSGP	NP_690584.1	297	367

Conventions are the same as in Table 2. uncl: unclassified.

Abreviations: N, nucleoprotein; P, phosphoprotein; NS, non structural protein (phosphoprotein); NSs, non structural protein produced from small RNA; SGP, structural glycoprotein; sSGP, soluble structural glycoprotein

(a) Several proteins are not mentioned in the corresponding genome file and thus have no accession number, although their existence has been proven (see Material and Methods). We provide their sequence in supplementary File S1.

1 **Table 7. Pairs of recognizable ancestral/novel overlapping protein regions**

Family (*-viridae*)	Genus (*-virus*)	Protein	Novel region Ancestral region (aa)	Matching PFAM families [Matching PFAM clans]	Suggested new families [Suggested new clans]	Common name of corresponding region	Function of novel full-length protein Function of ancestral full-length protein	Function of novel region
Birna	*Avibirna* *(+aquabirna)*	VP5	28-149	Birna_VP5			Anti-apoptosis	Anti-apoptosis (36)
		VP2	13-134	Birna_VP2 [Viral_ssRNA_CP]		Capsid protein with a Nucleoplasmin-like fold	Viral capsid	
Bunya	*Orthobunya*	NSs	60-98	Bunya_NS-S			Suppressor of RNA silencing; Inhibitor of interferon response Inhibitor of viral polymerase	nd
		N	66-104	Bunya_nucleocap, Tenui_N Phlebovirus_N	[Bunyaviridae_N]	N-terminal moiety of nucleoprotein of *Bunyaviridae* and *Tenuiviruses*	Binds to and protects the viral genome	
Flexi	*Potex*	TGBp3	10-50	7kD_coat			Virus cell-to-cell movement	Virus cell-to-cell movement (48)
		TGBp2	72-112	Plant_vir_prot		Movement protein	Virus cell-to-cell movement	
	Tricho	Movement protein	383-460	-			Virus cell to cell movement	nd
		Coat protein	28-105	Flexi_CP, Clostero_coat, Tricho_coat, Poty_Coat	[Flexuous_coat]	Coat protein of flexuous viruses	Viral capsid	
	Mandari	NABP	1-53	-			nd	nd
		Coat protein	226-278	Flexi_CP, Clostero_coat, Tricho_coat, Poty_Coat	[Flexuous_coat]	Coat protein of flexuous viruses	Viral capsid	
	Capillo	Polyprotein	1593-1840	-			Multifunctional viral replicase	nd
		Movement protein	10-257	MP, 3A, TBSV_P22	[30K_MP]	Movement protein of the 30K superfamily	Virus cell-to-cell movement	
Hepadna	*Ortho-hepadna*	L	193-427	vMSA			Viral envelope	Viral envelope (7)
		P	380-614	RVT_1 [RVT]		Reverse Transcriptase domain	Reverse transcriptase	
		X	1-39	X			multifunctional regulator of transcription, cell cycle, and apoptosis	nd
		P	793-831	DNA_pol_viral_C, Transposase 36	[RNAse_H] [(1)]	RNAse H	DNA/RNA duplex endoribonuclease	
Tetra	*Betatetra*	Replicase	1398-1851	-			Multifuntional viral replicase	nd
		Coat protein	83-536	Peptidase_A21, Peptidase_A6 [Viral_ssRNA_CP]		Capsid protein	Viral capsid Self-cleaving peptidase	
	Omegatetra	P17	119-158	-			Nd	nd
		Coat protein	88-127	Peptidase_A21, Peptidase_A6 [Viral_ssRNA_CP]		Capsid protein	Viral capsid	

Table 7 (continued)

Family (-viridae)	*Genus (-virus)*	Protein	Novel region Ancestral region (aa)	Matching PFAM families [Matching PFAM clans]	Suggested new families [Suggested new clans]	Common name of corresponding region	Function of protein	Function of novel region
Tombus	*Tombus (+aureus)*	P19	20-148	Tombus_p19			Suppressor of RNA silencing	Suppressor of RNA silencing (80)
		P22	30-158	TBSV_P22, MP, 3A	[30K_MP]	Movement protein of the 30K superfamily	Cell-to-cell movement of viral RNA	
	Carmo	P23	45-169	-			Factor indispensable for host-specific replication (54)	nd
		Replicase	48-172	Tombus_P33, Luteo_P1-P2	[Tombus_Luteo_P33]	P33 auxiliary replication protein	Essential component of viral replicase	
		P25	77-224	-			Long distance (systemic) movement of viral RNA	Long distance (systemic) movement of viral RNA (119)
		Coat	81-228	Viral_coat [Viral_ssRNA_CP]		Capsid protein with a Nucleoplasmin-like fold	Encapsidation of virion	
	Machlomo	P31	175-279	-			nd	nd
		Coat protein	46-150	Viral_coat [Viral_ssRNA_CP]		Capsid protein with a Nucleoplasmin-like fold	Encapsidation of virion	
Tubi	*Pomo (+Peclu, Hordei)*	TGBp3	1-38	Viral_Beta_CD			Cell-to-cell movement of viral RNA	nd
		TGBp2	72-109	Plant_vir_prot		Movement protein	Cell-to-cell movement of viral RNA	
Tymo	*Tymo*	Movement protein	56-332	Tymo_45kd_70kd			Long distance (systemic) movement of viral RNA	Long distance (systemic) movement of viral RNA (12)
		Replicase	58-219	Methyltrans_Typ, Vmethyltransf	Typovirus_MT_GT_N [Alphaviruslike_MT_GT_N]	N-terminal moiety of the MT-GT of these viruses	MT-GT	
			220-334	Methyltrans_Typ, Vmethyltransf	Typovirus_MT_GT_C [Alphaviruslike_MT_GT_C]	C-terminal moiety of the MT-GT of these viruses ("Y region")	MT-GT	
Uncl.	*Umbra*	RNP	6-237	Umbravirus_LDM			Long distance (systemic) movement	Long distance (systemic) movement (91)
		Movement protein	1-232	3A, MP, TBSV_P22	[30K_MP]	Movement protein of the 30K superfamily	Virus cell-to-cell movement	

Caption of Table 7 (previous page)

Abbreviations are the same as in Fig. 4. nd: not determined.

For each pair of overlapping protein regions, we indicate the boundaries of the ancestral and of the novel region (respectively below and above). When several genera encode homologous overlaps, only one is presented and the others are given between brackets (e.g. *pomoviruses, pecluviruses and hordeiviruses* encode a homologous TGBp2/TGBp3 overlap but only the data for *pomoviruses* are presented).

We indicate PFAM families and clans that match these regions, and proposed ones suggested on the base of profile-profile comparisons. Note that the boundaries of ancestral regions might extend beyond those of the corresponding PFAM family since the former have been determined structured-based methods in addition to sequence-based methods. The "species" function at http://pfam.sanger.ac.uk can be accessed for the taxonomic distribution of each PFAM family.

We indicate the function of full-length ancestral and novel proteins (bibliographical information can be found on the PFAM web site above), and the specific function of novel regions, when available.

(1) We suggest that the related families DNA_pol_viral_C and Transposase_36 are part of the existing clan RNAse_H on the basis of significant similarity of Transposase_36 to a member of this clan, the endonuclease family DDE, established through profile-profile comparison. This is coherent with an earlier report (57).

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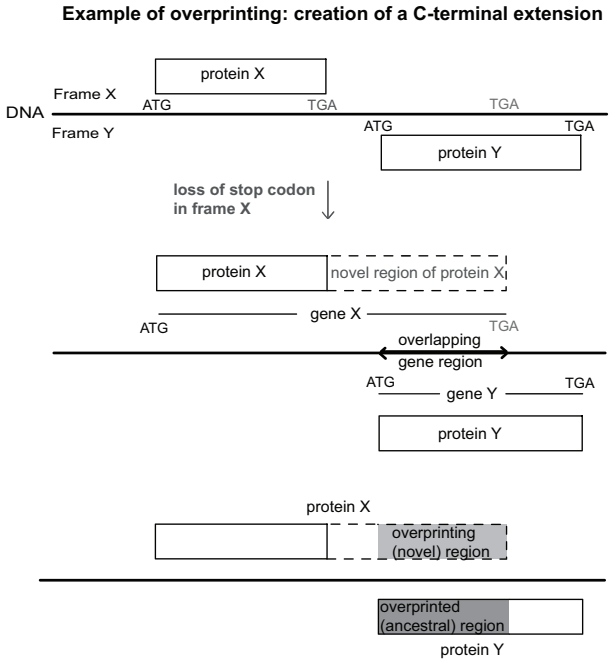

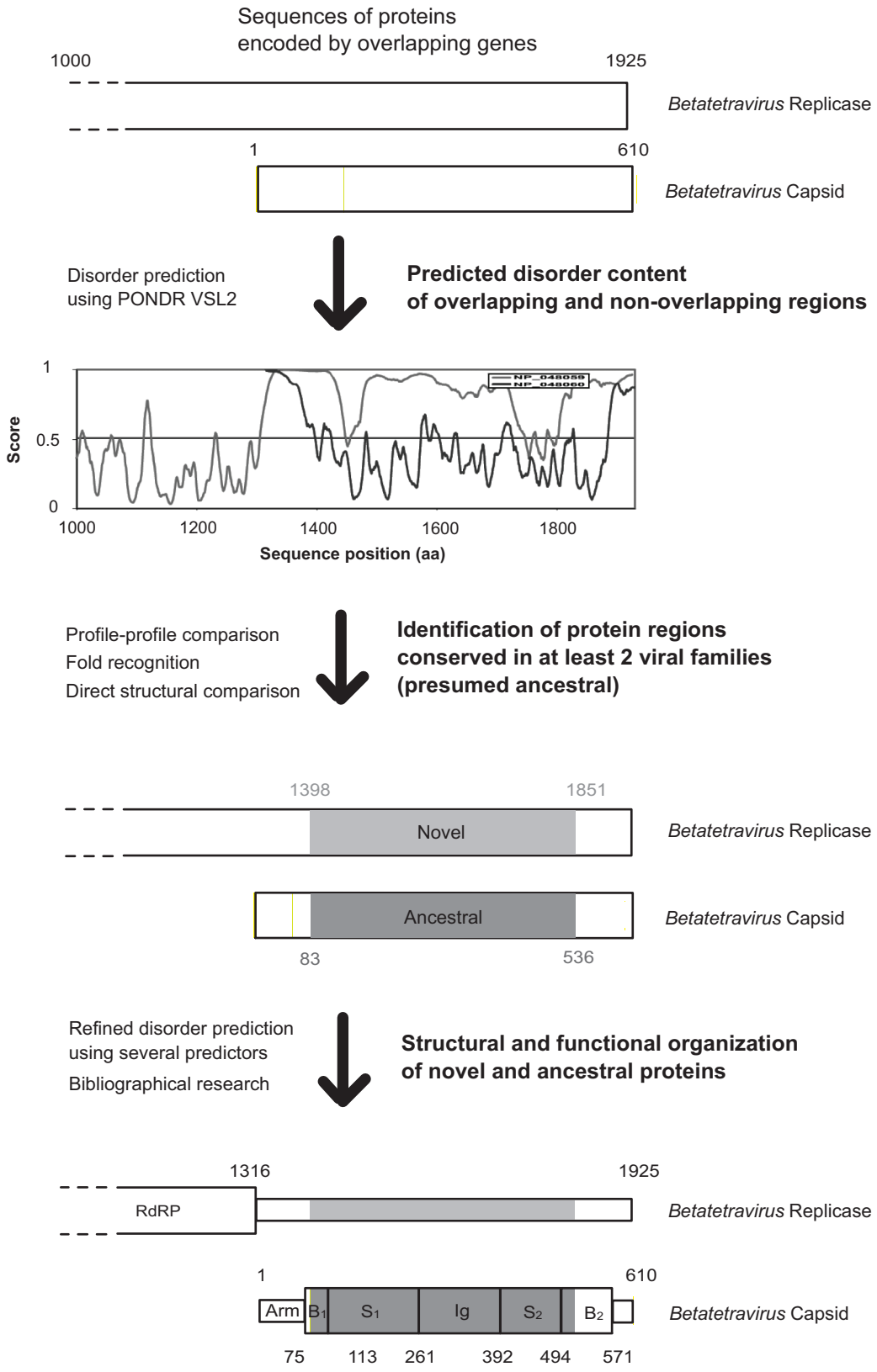

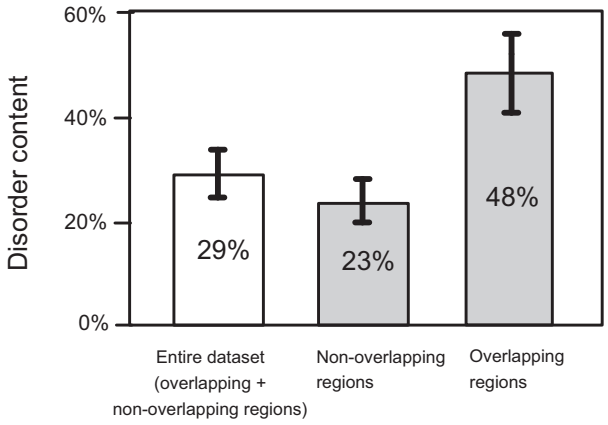

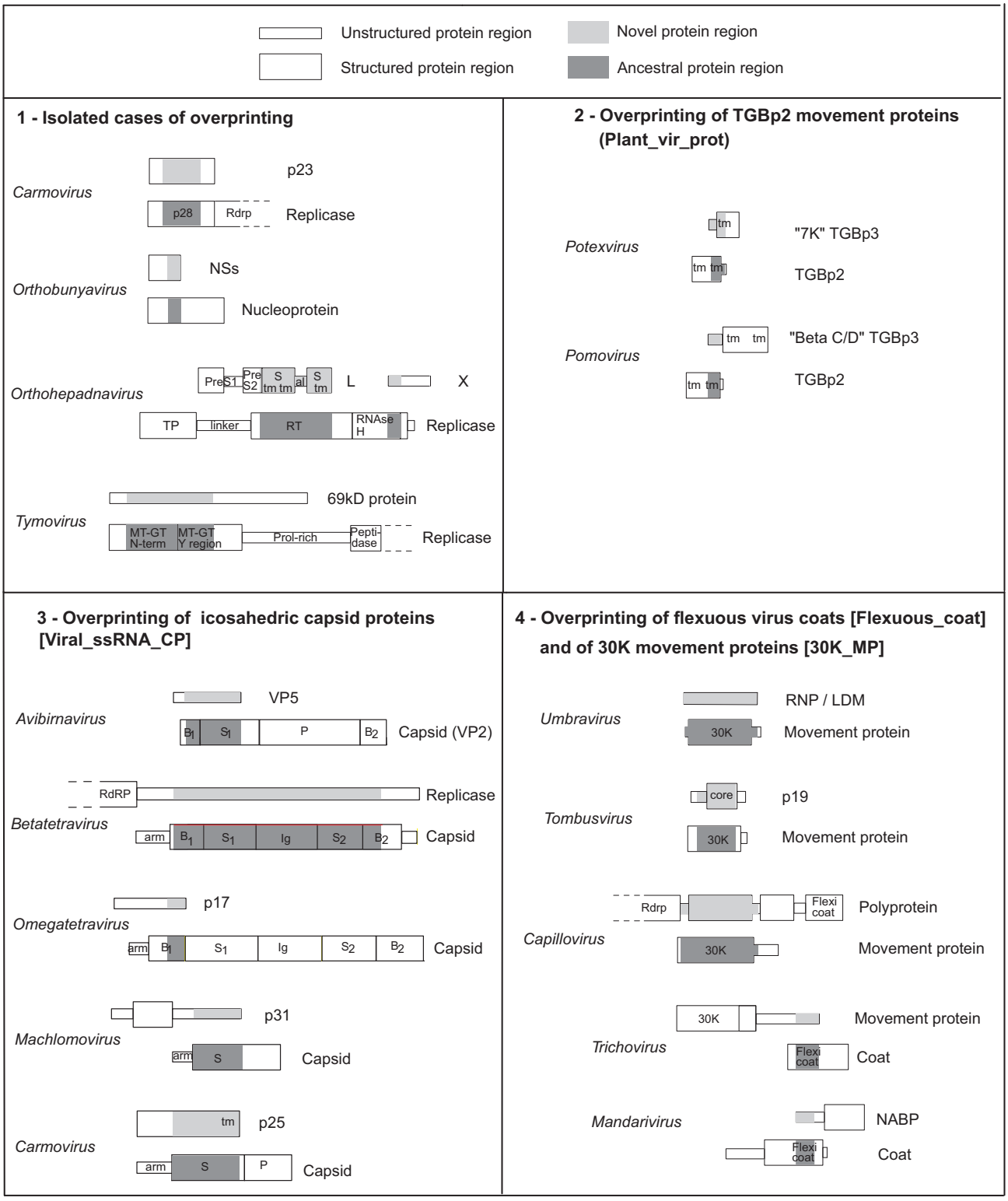

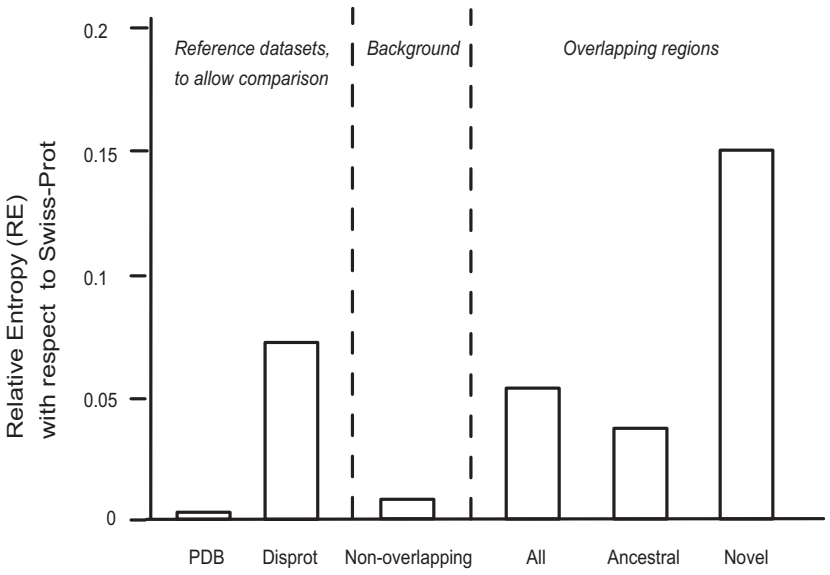

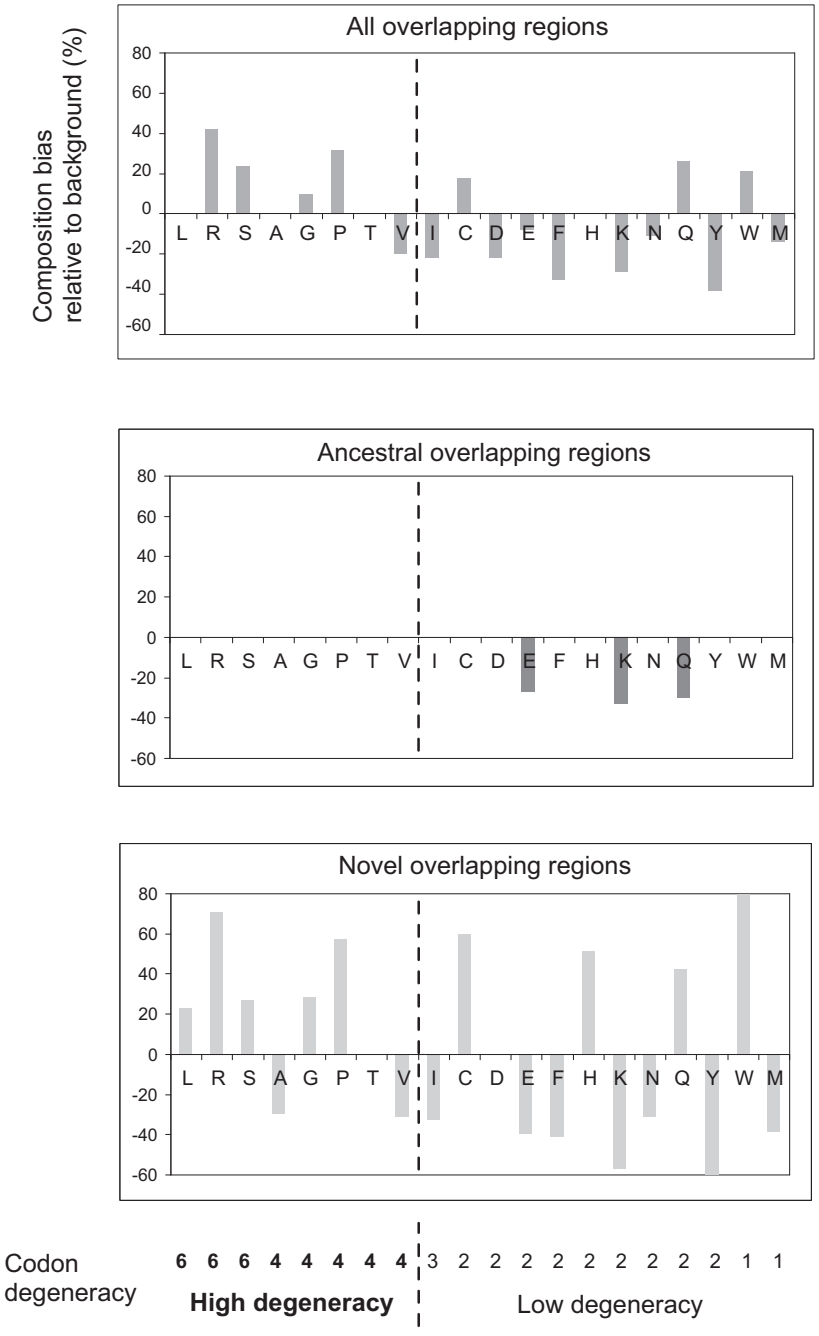

Enriched in disorder-promoting aa

Depleted in order-promoting aa

($P<10^{-6}$)

-

Enriched in disorder-promoting aa

Depleted in order-promoting aa

($P<10^{-5}$)